Prostate Apoptosis Response-4 (PAR4) Suppresses Growth and Invasion of Breast Cancer Cells and Is Positively Associated with Patient Survival

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Abstract. *Background: Prostate apoptosis response-4 (PAR4) plays an important role in apoptosis and survival of cancer cells. The current study aimed to further elucidate its role in breast cancer. Materials and Methods: PAR4 expression in human breast cancer tissue was examined using quantitative polymerase chain reaction (qPCR) and immunohistochemical staining (IHC). Plasmids containing full-length human PAR4 coding sequence were used to overexpress PAR4 in breast cancer cells and the effect on cellular functions was examined using both in vitro functional assays and an in vivo murine model. Results: Patients with low PAR4 transcript levels had poorer overall survival. PAR4 expression may be associated with differential expression of oestrogen receptors α and β in the tumours. Overexpression of PAR4 in MDA-MB-231 cells resulted in reduced cell growth and invasion, and also reduced in vivo tumour growth. Conclusion: Decreased PAR4 expression in breast cancer is associated with shorter survival. PAR4 suppresses growth and invasiveness of breast cancer cells.*

Prostate apoptosis response-4 (*PAR4*), also known as protein kinase C (PKC) apoptosis Wilms tumour 1 (WT1) regulator (*PAWR*), was initially identified in prostate cancer cells undergoing apoptosis in response to ionomycin (1). The PAR4 protein comprises a leucine zipper domain (LZ) at the carboxyl terminal region, two nuclear localisation sequences (NLS1 and NLS2) at the *N*-terminal region, a nuclear export sequence and a selective for apoptosis of cancer cells domain (SAC), that is unique to the PAR4 protein. Nuclear localisation sequence 2 is essential for the translocation of PAR4 into the cell nucleus and subsequent suppression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. A similar function has not been observed in *NLS1* deletion cells (2). The PAR4 protein can form homo- and hetero- dimers *via* the LZ domain, which also mediates binding with RAC-alpha serine/threonine-protein kinase (AKT1) leading to AKT1-induced phosphorylation and inhibition of PAR4 (3). WT1, death-associated protein-like kinase and ζPKC can all interact with PAR4 *via* the LZ domain to form heterodimers (4-6). PAR4 interacts with atypical isoforms of PKC, such as ζPKC and λPKC *via* their regulatory domains to inhibit their enzymatic activity (5). For example, PAR4 can inhibit activation of PKC by phosphatidylserine, which is the classical activator of all PKCs (5).

PAR4 sensitises cells to apoptosis induced by tumour necrosis factor (TNF), doxorubicin, etoposide, UV irradiation, growth-factor deprivation, and ionizing radiation (7, 8). PAR4 can selectively induce apoptosis of cancer cells, but not, or only to a lesser degree, of normal cells, and it is thought that the SAC domain of PAR4 and protein kinase A (PKA) play pivotal roles in this process (2, 9). Higher endogenous PKA levels expressed by cancer cells can sensitise the cells to the PAR4-mediated apoptosis, and phosphorylation of the SAC domain of PAR4 in cancer cells by PKA has been shown to be critical for apoptosis (9, 10). On the other hand, endogenous PAR4 can be phosphorylated and inactivated by AKT, resulting in inhibition of apoptosis and this may be at least one of the reasons why cancer cells are able to survive in the presence of endogenous PAR4 (3). Apart from direct suppression of the NF-κB pathway, PAR4 can also sequester topoisomerase 1, thereby preventing DNA unwinding and inhibiting cell-cycle progression, transcription and cellular transformation (11). In addition to its intracellular activities, PAR4 can also be secreted with the assistance of a protein called glucose-regulated protein 78 (GRP78), which is an endoplasmic reticulum (ER)

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Table I. *Prostate apoptosis response-4 (PAR4)/cytokeratin 19 (CK19) transcript levels in breast cancer.*

Clinical/pathological feature		No. of samples	Median (copy no.)	IQR	*p*-Value
Tissue	Normal	21	75	0-1776	0.548
	Tumour	91	23	0-690	
Grade of differentiation	Well	11	0	0-163	
	Moderate	31	1	0.324	0.797
	Poor	48	134	0-3000	0.070
NPI group	1 (<3.5)	44	11	0-363	
	2 (3.5-5.4)	30	12	0-3035	0.987
	3 (>5.4)	13	223	0-2466	0.648
Overall stage of disease	I	47	1	0-124	
	II	31	206	0-5786	0.003*
	III	5	84	0-2909	0.368
	IV	4	1.1	0.1-281.1	0.972
Clinical outcome	Disease-free	63	10	0-481	
	Poor outcome				
	With Metastasis	5	83	2-23206	
	With local recurrence	4	1026	0-1964	0.823
	Died of breast cancer	14	14	0-2051	0.467
	Bone metastasis	7	37.6	0-319.5	0.837
Histology	Ductal	71	40	0-690	0.178
	Lobular	11	0	0-5711	
	Others	5	37.6	0.4-184.6	0.917
ERα	Negative	57	106	0-2034	0.005*
	Positive	30	0	0-114	
ERβ	Negative	69	5	0-357	0.047*
	Positive	20	451	1-5073	
Lymph node status	Negative	44	11	0-363	0.852
	Positive	43	38	0-2142	

*Statistically significant difference ($p<0.05$). IQR: Interquartile range, NPI: Nottingham Prognostic Index, ER: oestrogen receptor.

protein. GRP78 is a chaperone protein involved in protein folding and regulation of ER stress signalling (12). Under ER stress, intracellular PAR4 binds to GRP78 and is subsequently translocated from the ER to the plasma membrane, where GRP78 acts as a receptor for PAR4 (13). Upon the binding of cell-surface GRP78 with extracellular PAR4, the adapter protein FAS-associated death domain recruits caspase-8, leading to activation of extrinsic apoptotic cascades (13).

Since the discovery of PAR4 in prostate cancer cells, the selective pro-apoptotic effects of PAR4 on cancer cells have been investigated in relation to several other malignancies. Previous studies have suggested that PAR4 acts as a tumour suppressor in renal cancer, neuroblastoma, acute and chronic forms of leukaemia, breast cancer, pancreatic cancer and lung cancer (14-19). In a previous study, we demonstrated that PAR4 plays a key role in bone morphogenetic protein 9-induced apoptosis in prostate cancer cells (20). The current study examined the expression of PAR4 in breast cancer tissues and its effect on both *in vitro* and *in vivo* growth of breast cancer cells.

Materials and Methods

Materials. Human breast cancer cell lines MDA-MB-231, MCF7 and ZR-75-1 were purchased from the European Collection of Cell Cultures (Salisbury, UK). The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 10% foetal calf serum and antibiotics. Monoclonal mouse anti-PAR4 was obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA). Unless otherwise stated, other materials and reagents were purchased from Sigma-Aldrich Ltd (Poole, Dorset, UK).

Collection of breast tissue samples. Breast cancer tissues (n=127) and normal background breast tissues (n=34) were collected during surgery and stored at −80˚C until use. All patients were followed-up clinically after surgery, with a median follow-up period of 120 months (June 2004). Histopathological details were obtained from pathological reports and confirmed by a consultant pathologist (Table I). All protocols were reviewed and approved by the Bro Taf Health Authority local Research Ethics Committee and patients participated with informed consent.

RNA extraction, reverse transcription polymerase chain reaction (RT-PCR) and quantitative PCR. Total RNA was extracted from

Table II. *Primer sequences for polymerase chain reaction (PCR).*

Gene	Forward	Reverse
PAR4 qPCR	5'-GATCTTACGCTTCCCTTACC	5'-ACTGAACCTGACCGTACA ATGCCAGGAGACGACCTC
CK19 qPCR	5'-CAGGTCCGAGGTTACTGAC	5'-ACTGAACCTGACCGTACACCGTTTCTGCCAGTGTGTCTTC
PAR4	5'- GTCCTCTCTCCTCCCTTCTA	5'-CTGCCTCCTCTTCCTTCC
PAR4 exp	5'-ATGGGGCGGGCGGGCCGACTT	5'-TCAGGCCGCCCACCAGGGCTCCGG
GAPDH	5'-GGCTGCTTTTAACTCTGGTA	5'-ACTGTGGTCATGAGTCCTT

frozen tissues and cultured cells using TRI regent® (Sigma-Aldrich Ltd). The concentration of RNA was determined using a UV spectrophotometer (WPA UV 1101; Biotech Photometer, Cambridge, UK).

Reverse transcription was carried out using an RT kit with an oligo (dT) primer supplied by ABgene® (Life Technologies Ltd., Paisley, UK) using 0.5 μg of total RNA for each 20 μl RT reaction. The quality of cDNA was verified by examining a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Conventional PCR was performed with specific primers for *PAR4*. The conditions for the PCR were: 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 1 min, followed by a final extension for 7 min at 72˚C. The products were visualized on a 1.5% agarose gel after staining with ethidium bromide. PAR4 transcript levels in the breast tissue specimens were determined using real-time qPCR, based on the Amplifluor™ technology, as reported previously (21). Briefly, pairs of qPCR primers were similarly designed using the Beacon Designer™ software (PREMIER Biosoft, Palo Alto, CA, USA), but an additional sequence was added to the antisense primer. This is known as the Z sequence (5'-actgaacctgaccgtaca-3'), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK). The reaction was carried out using iCycler iQ™ (Bio-Rad, Hemel Hempstead, Hertfordshire, UK), which is equipped with an optical unit that allows real-time detection of 96 reactions. The levels of the *PAR4* transcript are reported here as the number of transcript copies per 50 ng RNA, generated from an internal standard, cytokeratin 19 (*CK19*) that was simultaneously amplified during the same qPCR. The sequences of the primers used in the current study are listed in Table II.

Immunohistochemical staining of PAR4. Frozen sections of breast tumours and background breast tissue were cut at a thickness of 6 μm. The sections were air-dried and fixed in a mixture of 50% acetone and 50% methanol. The sections were then placed in Tris-buffered saline buffer for 20 min to rehydrate. Sections were then incubated for 20 min in a 0.6% bovine serum albumin blocking solution and probed with the primary antibody to PAR4 (1:100 dilution) for 1 h at room temperature. Following extensive washing, sections were incubated for 30 min in the secondary biotinylated antibody (Multi Link Swine anti-goat/mouse/rabbit immunoglobulin; Dako Inc., Ely, Cambridgeshire, UK). Avidin/Biotin Complex (Vector Laboratories Ltd, Peterborough, UK) was then applied to the sections, followed by washing. The sections were subsequently incubated with the chromogen 3,3'-diaminobenzidine (Vector Laboratories Ltd) in the dark for 5 min, and were then counterstained in Gill's haematoxylin. The IHC stained sections were assessed by two researchers independently, the description of the PAR4 staining was agreed by both researchers with a final validation by a third researcher.

Western blot analysis. Cells were lysed in HCMF buffer containing 1% Triton, 0.1% sodium dodecyl sulfate, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml aprotinin for 30 min before centrifuging at 13,000 × *g* for 10 min. Protein concentrations were quantified using a spectrophotometer (BioTek™ ELx800™; BioTek, Swindon, UK). Equal amounts of each protein sample (20 μg/lane) were separated in a 10% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose sheets. After blocking in 10% skimmed milk for 60 min, the blots were probed with the antibody against PAR4 and peroxidase-conjugated secondary antibody. Protein bands were developed using the Supersignal™ West Dura system (Pierce Biotechnology, Inc., Rockford, IL, USA), and photographed with an UVITech imager (UVITech, Inc., Cambridge, UK).

Overexpression of PAR4 in MCF-7 and MDA-MB-231 cells. The PAR4 overexpression vectors were constructed using a pEF6/V5/His vector and used to transfect MCF-7 and MDA-MB-231 cells. Following transfection, cells were selected using blasticidin (5 μg/ml). MCF-7PAR4exp, MDA-MB-231PAR4exp that overexpressed *PAR4* and respective controls (MCF-7pEF and MDA-MB-231pEF), which carried empty plasmid vectors, were established. MCF-7WT and MDA-MB-231WT were designated for the wild-type cells.

In vitro cell growth assay. A standard procedure was used as previously described (20, 21). Cells were plated into a 96-well plate (2,500 cells/well). Cell growth was assessed after 1, 3 and 5 days. Crystal violet was used to stain cells, and absorbance was determined at a wavelength of 540 nm using a spectrophotometer (BioTek™ ELx800™; BioTek).

In vitro invasion assay. According to a standard procedure, transwell inserts with 8 μm pore size were coated with 50 μg Matrigel (BD Matrigel™ Basement Membrane Matrix; BD Bioscience, Oxford, UK) and air-dried. After rehydration, 20,000 cells were added to each well. After 96 hours, cells that had migrated through the matrix to the other side of the insert were fixed, stained and then counted under a microscope.

Cell-matrix adhesion assay. As previously described (22), 40,000 cells were added to each well of a 96-well plate which was pre-coated with Matrigel™ (5 μg/well). After 40 min of incubation, non-adherent cells were washed-off using balanced salt solution buffer. The remaining adhered cells were fixed, stained and then counted.

Tumour growth in an athymic mouse model. Female athymic nude mice (4-8 week old CD-1® Nude mice; Charles River Laboratories, Oxford, UK) were subcutaneously injected with MCF-7 breast cancer cells (1.0×10^6 cells) in Matrigel™ (2.5 mg/ml). The mice were kept in sterilised, filtered cages under 12-h dark/12-h light standardized environmental conditions. They were weighed twice weekly in accordance with Home Office regulations. Tumour size was measured twice a week using digital callipers and calculated as volume (mm^3)=0.512 × width^2 × length. This study was performed in strict accordance with the recommendations in the "Research and Testing Using Animals" guidance published by the UK Government Home Office (19). The study protocol and procedure (project licence no. 30/2591) were approved by the UK Government Home Office.

Statistical analysis. Statistical analyses were performed using Minitab® version 14 and SPSS version 18 statistical software packages (SPSS Inc., Chicago, IL, USA). Non-normally distributed data were assessed using the Mann–Whitney test, while the two sample *t*-test was used for normally distributed data. Kaplan–Meier survival analysis, Cox proportional hazards model and Spearman rank correlation coefficient were used to analyse data using SPSS. Differences were considered to be statistically significant at $p<0.05$.

Results

Perturbed expression of PAR4 in breast cancer. The expression of *PAR4* was examined in both breast tissue samples obtained from the clinical cohort and breast cancer cell lines. PAR4 was expressed at lower levels in all three breast cancer cell lines, *i.e.* MDA-MB-231, MCF-7 and ZR-75-1, compared to its expression in prostate tumour, which was used as a positive control (Figure 1A). The expression of PAR4 was also examined in a cohort of human breast cancer tumour samples using both qPCR and immunohistochemical staining. PAR4 staining was seen in cytoplasm of both mammary epithelial cells and cancer cells in the tissues. The staining of PAR4 was much weaker or absent from the breast cancer cells compared to the staining seen in mammary epithelial cells (Figure 1B). After the quantification of *PAR4* transcripts in the breast tissues, the *PAR4* transcript levels were normalised against the corresponding *CK19* transcript levels. Although a similar trend was seen, no significant difference was found in the transcript levels detected in the tumours compared to the normal mammary glands (Table II).

Association of PAR4 transcript levels with clinical and histopathological features of breast cancer. There was a trend towards increased *PAR4* expression in poorly-differentiated tumours compared to well-differentiated ($p=0.070$) and moderately-differentiated ($p=0.013$, comparison not shown in Table) tumours. Although *PAR4* transcript levels varied between tumours of different histological type (*i.e.* ductal *vs.* lobular *vs.* others) these differences were not statistically significant. When analyzed according to overall tumour stage, stage II breast cancer exhibited a significantly higher level of *PAR4* compared stage I ($p=0.003$) and stage IV ($p=0.108$, comparison not shown in Table) cancers. Significantly reduced *PAR4* expression was seen in oestrogen receptor α (ERα)-positive tumours ($p=0.005$), while significantly increased expression was seen in ERβ-positive tumours ($p=0.047$) (Table I).

The prognostic significance of *PAR4* transcript level was assessed in accordance with the Nottingham Prognostic Index (NPI) scores of the patients. NPI-1 (NPI score ≤3.4; n=48) indicates good prognosis, NPI-2 (NPI score >3.4 and ≤5.4; n=32) indicates moderate prognosis, whilst NPI-3 (NPI score >5.4; n=13) indicates a poor prognosis. The *PAR4* transcript level was higher in the NPI-3 group compared to NPI-1 and NPI-2 groups, but these differences did not reach statistical significance.

Kaplan–Meier survival analysis indicated that overall survival was significantly shorter in patients with low *PAR4* transcript levels compared to patients who had higher *PAR4* expression ($p=0.0082$). The average level of *PAR4* transcripts in NPI2 group tumours was used as a threshold for this analysis. Patients with higher expression also had longer disease-free survival [median=133.1 months, 95% confidence interval (CI)=121.1-145.1 months] compared to patients who had lower expression (118.4 months, 95% CI=103.2-133.5 months) ($p=0.0597$) (Figure 1C and D).

PAR4 regulates adhesion and invasion of breast cancer cells. Constructed plasmid vectors carrying the *PAR4* coding sequence were transfected into two breast cancer cell lines (MCF-7 and MDA-MB-231). Increased expression of *PAR4* was verified in the transfected cells (Figure 2A). Adhesion to an artificial matrix (Matrigel™) was used to assess the effect of *PAR4* overexpression on the adhesiveness of the cancer cells. *PAR4* overexpression resulted in reduced adhesion of MCF-7 cells compared with control cells. A similar effect was also seen in the MDA-MB-231 cells but to a lesser extent (Figure 2B). We also determined the influence on invasiveness using a transwell model. *PAR4* overexpression inhibited the invasion of both cancer cell lines (Figure 2C).

PAR4 and growth of breast cancer cells. Overexpression of *PAR4* in MDA-MB-231 cells (MDA-MB-231PAR4exp) resulted in a reduction of cell growth *in vitro* (Figure 3). Such an inhibitory effect was not seen in the MCF-7 cells. The influence of *PAR4* on growth of breast cancer cells was further examined in an athymic mouse tumour model. The overexpression of *PAR4* reduced the rate of *in vivo* tumour growth compared to the control group ($p<0.05$).

Discussion

Our data suggest that the level of *PAR4* expression is generally lower in breast cancer tissue compared to normal

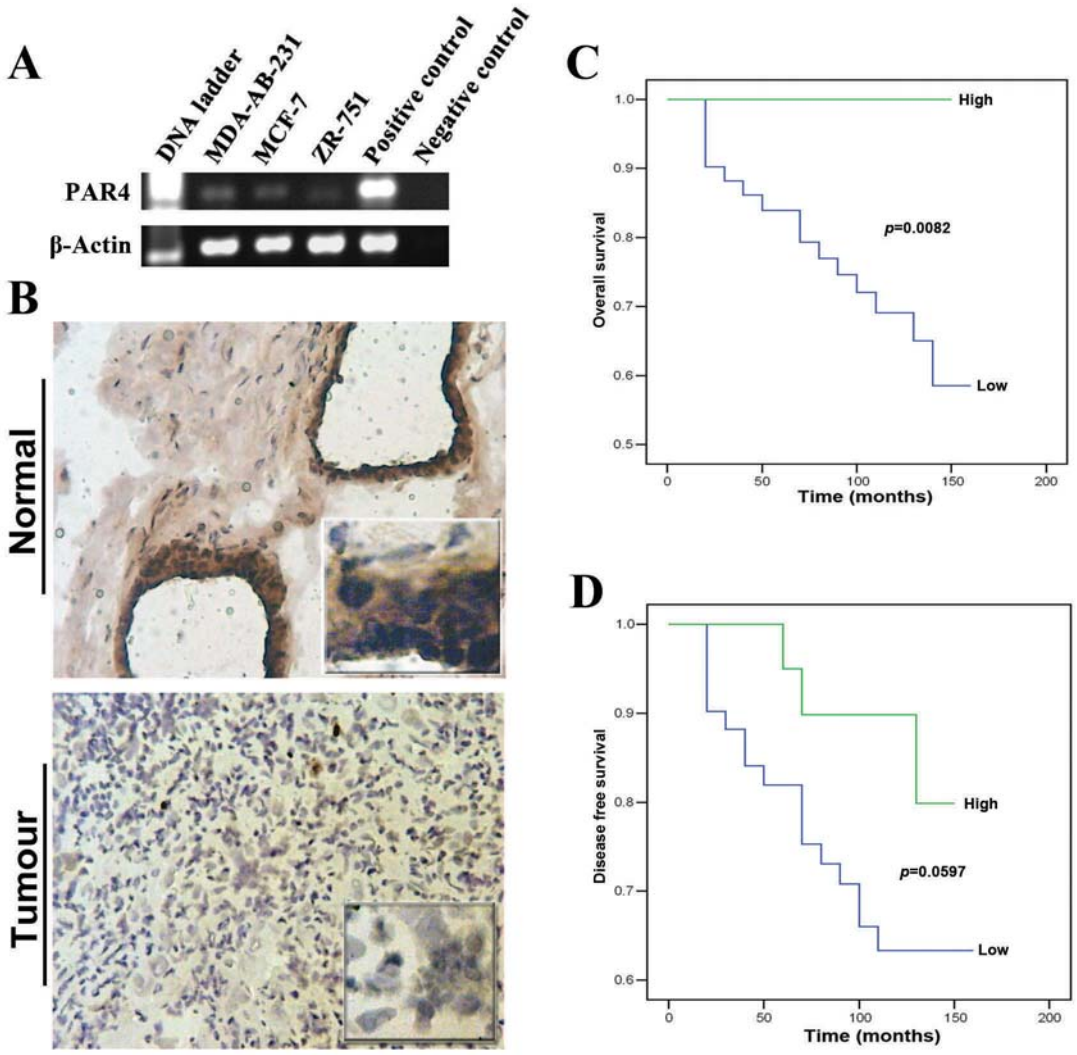

Figure 1. *Expression of prostate apoptosis response-4 (PAR4) in breast cancer and its association with patient survival. A: Expression of PAR4 transcripts in breast cancer cell lines, MDA-MB-231, and MCF-7, and mammary epithelial cells ZR-75-1 compared to prostate cancer as a positive control. B: Immunohistochemical staining of PAR4 protein in breast cancer tissue compared to normal breast tissue. Images shown are taken at a magnification of ×100 and ×400 for the inserted panels. C: Overall survival of patients with breast cancer cohort patients according to PAR4 transcript level. D. Disease-free survival of patients with breast cancer according to PAR4 transcript level. The average level of PAR4 transcripts in NPI2 group tumours was used as threshold for the survival analysis.*

breast tissue, although the difference in transcript level observed during qPCR failed to reach statistical significance. This is in keeping with previous studies, which have suggested that PAR4 acts as a tumour suppressor in a range of malignancies, including breast cancer (14-17, 19, 23). Previous studies investigating PAR4 expression in primary breast cancer tissue samples suggest that around 35-43% are positive for PAR4 expression compared to none of normal breast tissue samples (17, 24). In contrast to our results, other studies have found higher levels of PAR4 protein expression in breast cancer cell lines compared to normal breast cell lines (25). Mendez-Lopez *et al*. also found that there was strong nuclear PAR4 expression in benign lesions and breast cancer, mostly in epithelial and ductal cells, although PAR4 was absent from normal breast tissue (26).

With regard to localisation of PAR4 expression within breast cancer cells, our data confirm the results of previous studies in breast and endometrial cancer which found that approximately 90% of PAR4 is localised to the cytoplasm (17, 27). El-Guendy *et al*. found that nuclear presence of

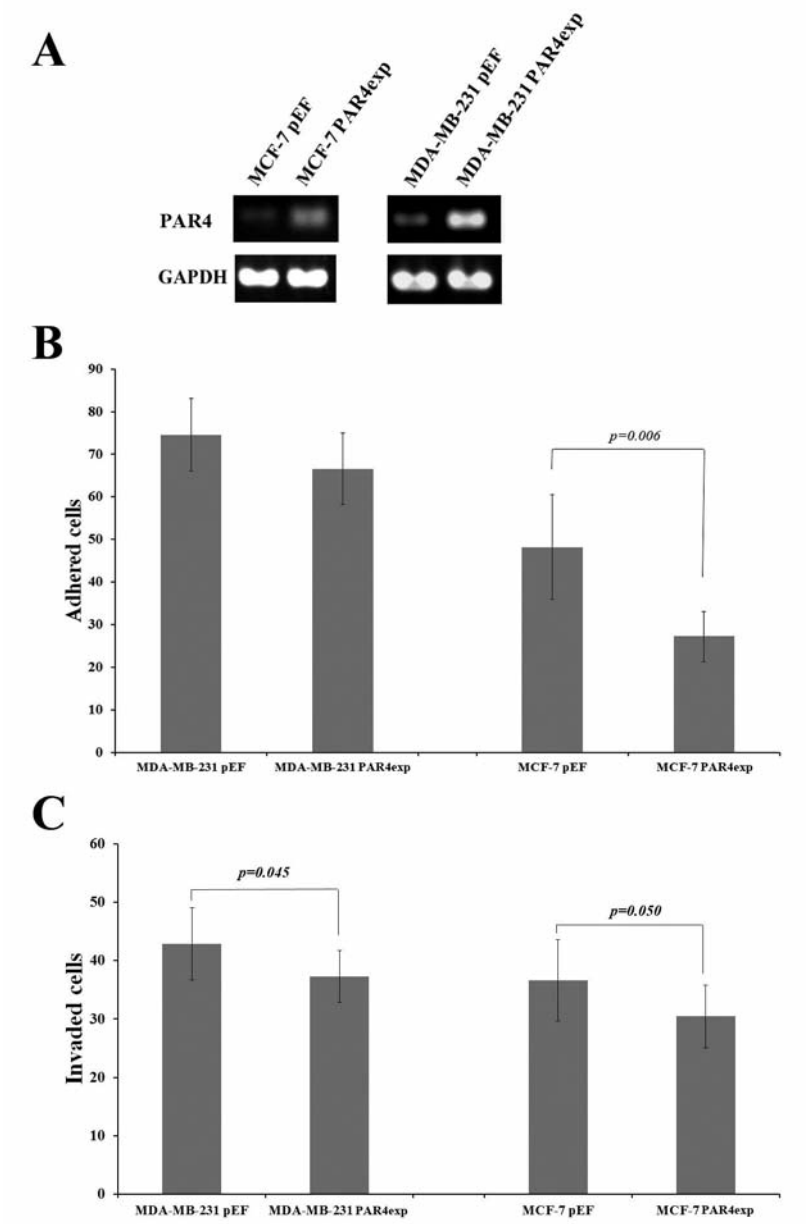

Figure 2. *Overexpression of prostate apoptosis response-4 (PAR4) in breast cancer cells and effect on cell adhesion and invasion. A: Expression of PAR4 in the transfected (PAR4exp) breast cancer cells was verified using reverse transcription polymerase chain reaction. B: Effect of PAR4 expression on adhesion of breast cancer cells to an artificial extracellular matrix. C: PAR4 overexpression was associated with reduced invasion by breast cancer cells.*

PAR4 is necessary for its ability to induce apoptosis and, unlike normal prostate cells where PAR4 is found in both cytoplasm and nucleus, they too found that PAR4 is restricted to the cytoplasm in prostate cancer cells (2).

We observed a trend towards higher *PAR4* expression in more poorly differentiated tumours, stage II disease and with increasing NPI score but this did not result in poorer outcomes and in fact, overall survival was significantly shorter in patients with low *PAR4* transcript levels. Several other authors have also found that low PAR4 expression is associated with poorer survival outcomes in patients with breast cancer (24, 26). Moreover, lack of nuclear PAR4 expression is associated with worsening of outcomes in patients with poor prognosis human epidermal growth factor receptor-positive, epidermal growth factor receptor-positive and pAKT-positive breast cancers (24).

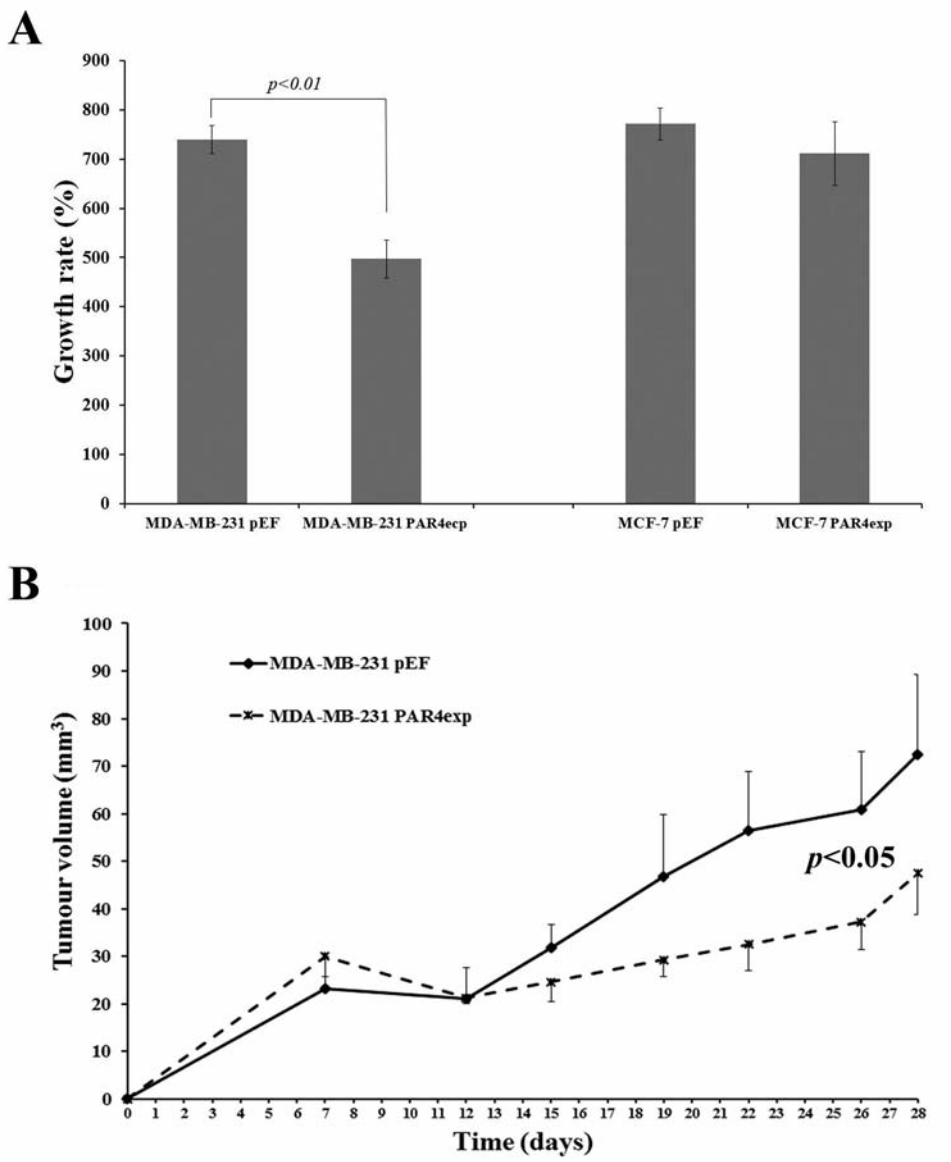

Figure 3. *Prostate apoptosis response-4 (PAR4) and breast cancer cell growth. A: PAR4 overexpression (PAR4exp) inhibited the in-vitro growth of MDA-MB-231 cells. B: Influence of PAR4 overexpression on the in-vivo growth of MDA-MB-231 cells in a murine model.*

Oncological therapy for breast cancer results in PAR4-dependent apoptosis of breast cancer cells *via* activation of ZIP kinase and macrophage galactose N-acetyl-galactosamine specific lectin 2 activation and subsequent multinucleation (28). However, it has been suggested that down-regulation of *PAR4* levels allows primary and metastatic breast cancer cells to evade this apoptotic response to therapy, resulting in tumour chemoresistance and local or distant disease recurrence (28, 29).

The *PAR4* expression level does not appear to vary with different histological types of breast tumour but higher PAR4 expression was significantly associated with ERα-negative and ERβ-positive tumours. Other authors reported that PAR4 expression was significantly inversely associated with progesterone receptor expression but did not find any association with ER status in breast cancer, although *PAR4* expression is known to be down-regulated by 17β-oestradiol *via* activation of ER (17, 30). In endometrial cancer, an association between down-regulation of *PAR4* and ER-positive status was shown (27).

The results of our functional assays investigating the effect of *PAR4* on breast cancer cell function suggest that *PAR4*

overexpression is associated with a reduction in cancer cell adhesion, invasion, cell proliferation and tumour growth in both *in vivo* and *in vitro* models. Consistent with these findings, Pereira *et al*. found that overexpression of *PAR4* in MCF-7 cells resulted in reduced cell proliferation whilst *PAR4*-knockdown increased MCF-7 cell proliferation (31). Interestingly, we did not observe this effect in MCF-7 cells but did see reduced growth of MDA-MB-231 cells and in tumour of our *in vivo* murine models. Previous work by Chaudhry *et al*. found that transforming growth factor-β (TGFβ), which is known to induce epithelial-to-mesenchymal transition involved in cell motility and metastasis, up-regulates *PAR4* expression and promotes its translocation to the nucleus *via* Smad (Sma, small family member identified in Caenorhabditis elegans and MAD, mothers against decapentaplegic homolog) and NF-κB pathways (32). They found that overexpression of *PAR4* is associated with increased cell migration, whilst silencing of its expression abrogates TGFβ-induced epithelial-to-mesenchymal transition (32). Previous authors have suggested that loss of cell adhesion and growth factor deprivation may actually induce *PAR4* expression, resulting in increased cell susceptibility to apoptosis (33).

In summary, our data suggest that *PAR4* expression is down-regulated in breast cancer and is significantly associated with poorer overall survival of patients with breast cancer. Data from *in vitro* and *in vivo* functional assays suggest that *PAR4* overexpression reduces cancer cell adhesion, invasion and growth and may, therefore, present a potential target for future novel therapies.

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